REMARKS

Claims 75-80 are presented. No new matter is involved.

The sections of the Office Action entitled "Continuation Data" and "Substitute Drawing" require no comment.

With respect to "Sequence Rule Compliance," this is the first time the particular issue set forth by the Examiner has been raised. Page 35 is amended. It now parallels U.S. Patent No. 6,599,699, to which the Examiner's attention is directed. No copy of the '699 patent is provided because it is lengthy, is available online, and was passed to issue by the Examiner of this application. Hence, submitting a copy of the patent is believed unnecessary.

With respect to the "Objection," applicants invoke their right to defer correction of the oath/declaration until allowance. This is permitted by the MPEP. Under the assumption that the Examiner is familiar with the MPEP, the relevant provisions have not been provided.

The Examiner has then rejected all claims under 35 U.S.C. § 112, first paragraph, arguing that the specification is only enabling for "an isolated cDNA sequence consisting of SEQ ID NO: 18." Applicants do not agree, and traverse.

While applicants do not agree that only cDNA is enabled, claim 75 and claims dependent thereon require this. Further claim 75 et seq. do require SEQ ID NO: 18, but use "comprising" language, and recite functional language, i.e., that the cDNA encodes a polypeptide which is processed intracellularly to a peptide encoded by nucleotides 70-96 of SEQ ID NO: 18. The peptide is described in U.S. Patent No. 5,405,940, referred to in prior correspondence. The '940 patent was incorporated by reference, in view of page 6, lines 22-24:

> "Specifically in U.S. patent application Serial Number 938,334, the disclosure of which is incorporated by reference...".

Turning to the specific objections raised by the Examiner, the Examiner has determined that she can ignore a prior USPTO determination that this peptide functions as a tumor rejection antigen. Patents are presumed enabled, and the '940 patent expressly states and claims that the peptide referred to *supra* is a tumor rejection antigen.

The peptide the nucleotides 70-96 of SEQ ID NO: 18 encodes is EVDPIGHVY. See the attached paper by Benlalam, et al., *J. Immunol.*, 171:6283-6289 (2003), especially page 6286, second column.

What is noteworthy about these statements is that the MAGE-A6 peptide is expressly described as being cross reactive with the MAGE-A3 peptide - with which it differs by one amino acid at position 8.

The MAGE-A3 peptide provokes CTLs, and as noted, the MAGE-A6 peptide is cross reactive with the T cells. As such, the Examiner's argument based on differences of one amino acid are moot.

The fact is, the peptides from homologous regions of different MAGE molecules are shown to stimulate T cells. The Examiner agrees that the '940 patent teaches the MAGE-A1 homolog does so. U.S. Patent Nos. 6,552,180 and 5,686,068 show that MAGE-2 functions as a tumor rejection antigen precursor, and that peptides derived therefrom stimulate T cells. U.S. Patent Nos. 6,565,857 and 6,488,932, shows that MAGE-A3 derived peptides stimulate T cells. The paper by Benlalam cited herein shows that MAGE-A3 and MAGE-A6 both stimulate CTLs. Kobayashi, et al., *Tissue Antigens*, 62(5):426-432 (2003), (abstract only), teaches that the MAGE-4 peptide also provokes CTLs.

What this shows is that one considers the relevant art, one expects MAGE molecules, including MAGE-A6, to be processed to peptides presented by HLA molecules to form T cell provoking complexes.

Claims 75-80 require that the MAGE-A6 peptide be encoded. Hence, the Examiner's position as set forth at page 7 - and presumably relating to the prior three pages of discussion - is untenable.

Similarly, since claims 75-80 do not present language relating to complementarity, the argument is moot.

The Examiner has referred to the "comprising" language of claim 74, but since new claim 75 requires a specific peptide to be encoded, the argument is moot.

Indeed, it is noted that the Examiner clearly notes at page 4, that SEQ ID NO: 18 - which is cDNA for MAGE-6 - is part of a larger molecule. Hence, the Examiner's position is contrary to the extent it is understood.

Applicants do not follow the argument at page 9 et seq. The activity attributed to MAGE molecules is their processing to immunoreactive peptides. This is all that is required. Further, it is shown that the MAGE family behaves this way generally.

With respect to the argument at page 11 ("B"), since this language is not in the claims, the argument is moot.

With respect to the Examiner's argument regarding immunotherapy and *in vivo* use (page 13 et seq.), since this is not claimed, its relevance is not seen.

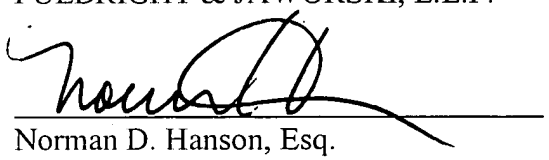
Turing to the rejection set forth at "C" at page 16, these arguments are moot, for the reasons given *supra*.

As to "D" at page 17, since "cDNA" is claimed, this argument is moot.

Allowance of the application is proper and is urged.

Respectfully submitted,

FULBRIGHT & JAWORSKI, L.L.P.



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Enclosures

Page 35, lines 13-18:

Example 32

Melanoma cell line LB-33-MEL was tested. Total mRNA from the cell line was used to prepare cDNA, which was then amplified with oligos CH09: (ACTCAGCTCCTCCCAGATTT nucleotides 4130-4146 of SEQ ID NO: 8), and CH010: (GAAGAGGAGGGGCCAAG) (SEQ ID NO: 27). These oligos correspond to regions of exon 3 that are common to previously described mage 1, 2, and 3.

1: Tissue Antigens. 2003 Nov;62(5):426-32.

New MAGE-4 antigenic peptide recognized by cytolytic T lymphocytes on HLA-A1 tumor cells.

Kobayashi T, Lonchay C, Colau D, Demotte N, Boon T, van der Bruggen P.

Kirin Brewery Co., Cellular Immunotherapy, Japan.

'Cancer-germline' genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in other normal tissues. They encode shared tumor-specific antigens, which have been used in small therapeutic vaccination trials of cancer patients. Gene MAGE-4, which is expressed in more than 50% of carcinomas of esophagus, head and neck, lung, and bladder, has two known alleles. Using PCR amplifications and digestions of the amplified product, we found that one third of the MAGE-4-positive samples expressed MAGE-4a. We folded HLA-A1 tetramers with peptide MAGE-4a169-177 EVDPASNTY, which is homologous to MAGE-1- and MAGE-3-encoded peptides recognized on HLA-A1 by cytolytic T lymphocytes. Blood lymphocytes from an individual without cancer were directly labelled with these A1/MAGE-4 tetramers. The very rare cells that were stained were sorted by flow cytometry and cloned. We isolated a cytolytic T-lymphocyte clone that lyzed specifically cells pulsed with this MAGE-4 peptide and HLA-A1 tumor cells expressing MAGE-4a, demonstrating that this antigenic peptide is processed efficiently in tumor cells. This peptide might therefore be useful for therapeutic antitumoral vaccination.

PMID: 14617050 [PubMed - in process]

Identification of Five New HLA-B*3501-Restricted Epitopes Derived from Common Melanoma-Associated Antigens, Spontaneously Recognized by Tumor-Infiltrating Lymphocytes[1]

Houssem Benlalam,* Boris Linard,* Yannik Guilloux,*† Agnès Moreau-Aubry,*† Laurent Derré,* Elisabeth Diez,* Brigitte Dreno,*‡ Francine Jotereau,*† and Nathalie Labarrière[2]*

We previously described HLA-B35-restricted melanoma tumor-infiltrating lymphocyte responses to frequently expressed melanoma-associated Ags: tyrosinase, Melan-A/MART-1, gp100, MAGE-A3/MAGE-A6, and NY-ESO-1. Using clones derived from these TIL, we identified in this study the corresponding epitopes. We show that five of these epitopes are new and that melanoma cells naturally present all the six epitopes. Interestingly, five of these epitopes correspond to or encompass melanoma-associated Ag epitopes presented in other HLA contexts, such as A2, A1, B51, and Cw3. In particular, the HLA-B35-restricted Melan-A epitope is mimicked by the peptide 26–35, already known as the most immunodominant melanoma epitope in the HLA-A*0201 context. Because this peptide lacked adequate anchor amino acid residues for efficient binding to HLA-B35, modified peptides were designed. Two of these analogues were found to induce higher PBL- and tumor-infiltrating lymphocyte-specific responses than the parental peptide, suggesting that they could be more immunogenic in HLA-B*3501 melanoma patients. These data have important implications for the formulation of polypeptide-based vaccines as well as for the monitoring of melanoma-specific CTL response in HLA-B*3501 melanoma patients. *The Journal of Immunology,* **2003, 171: 6283–6289.**

Characterization of tumor-associated Ag (TAA[3])-derived peptides efficiently presented by tumor cells and recognized by tumor-reactive CTL is critical for the development of TAA-specific T cell therapies. TAA identification has been especially effective for melanoma tumors, evidencing two major classes of melanoma-associated Ags (MAA): tumor-specific proteins unexpressed in normal tissues and melanocytic differentiation Ags, which are also expressed in normal melanocytes (for review, see Refs. 1 and 2). Most of these Ags are susceptible to provide multiple epitopes in different HLA contexts. To date, identifying HLA-A*0201-restricted epitopes has been a major goal for the development of vaccines useful for the largest fraction of melanoma patients. Thus, until now, most epitope-based immunotherapies have been performed in this HLA context (3–8). However, despite the induction of specific T cell responses, clinical efficacy of these assays remains disappointing. Tumor escape resulting from the induction of HLA or tumor epitope loss variants is often evoked as a possible reason for this limited effect (9, 10). The use of polyvalent vaccines composed of multiple tumor-associated peptides derived from different proteins and presented by various HLA molecules could at least partially counteract tumor escape. Hence, the identification of new epitopes from commonly expressed Ags restricted by various frequently expressed HLA is necessary.

Through a systematic screening of the specificity of 60 melanoma tumor-infiltrating lymphocyte (TIL) populations, we reported previously the recognition of ~20 new HLA-class I/peptide complexes, among which 6 were restricted by the HLA-B*3501 allele (Melan-A/MART-1, tyrosinase, gp-100, NY-ESO-1, and MAGE-A3/A6) (11). Because this HLA is one of the most frequently expressed HLA-B alleles (12), we sought to identify the B*3501-restricted epitopes recognized by these TIL populations and to look for the efficacy of their presentation by HLA-B35 melanoma cells.

Materials and Methods

Cell lines

Melanoma cell lines were established from fragments of metastasic tumors or tumor-invaded lymph nodes. Mouse fibrosarcoma WEHI 164 clone 13, used for TNF production assays and COS-7 cells were obtained from T. Boon (Ludwig Institute for Cancer Research (LICR), Brussels, Belgium). COS-7 cells, WEHI 164 clone 13, and melanoma cells lines were cultured, as described before (11). The EBV-B-transformed cell line LAZ 338 was a gift from T. Hercend (Vertex Pharmaceutical, Abingdon, U.K.). The BM36.1, an HLA-B*3501 TAP-deficient B-EBV cell line previously described (13), was a gift from A. Ziegler (Universitatsklinikum Charite, Berlin, Germany).

T cell clones

T cell clones were derived by limited dilution culture of polyclonal TIL obtained from melanoma-invaded lymph nodes of stage III patients (American Joint Committee on Cancer). TIL clones were cultured, as described before (11).

Synthetic peptides

Wild-type and modified peptides were purchased from EPYTOP (Nimes, France). Purity (>70%) was controlled by reversed-phase HPLC. Peptides were lyophilized, dissolved in DMSO at 10 mg/ml, and stored at −80°C.

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[3] Abbreviations used in this paper: TAA, tumor-associated Ag; DC, dendritic cell; MAA, melanoma-associated Ag; RA, relative affinity; TIL, tumor-infiltrating lymphocyte.

0022-1767/03/$02.00

HLA cDNAs

cDNAs coding for HLA-B*3501, HLA-B*3503, and HLA-B*3508 were obtained respectively in our laboratory from T. Boon (LICR) and from E. Zorn (Unité Institut National de la Santé et de la Recherche Médicale Unité 267, Villejuif, France). cDNAs coding for melanoma Ags MAGE-A3, MAGE-A6, tyrosinase, Melan-A/MART-1, and NY-ESO1/LAGE-2 were obtained from T. Boon. gp100 cDNA was cloned in our laboratory.

Construction of cDNA fragments

Tyrosinase fragments 1-1086 and 1-598 were kindly given by B. Van Den Eynde (LICR). cDNA restriction fragment of gp100 (1156–1986) was generated by enzymatic digestion with *Kpn*I. This fragment used, for expression, the internal ATG at position 1174. The other gp100 and tyrosinase cDNA fragments were obtained by PCR using specific oligonucleotides, and were cloned into pcDNA3 plasmid. Melan-A/MART-1 and NY-ESO-1 cDNA fragments were obtained by exonuclease III digestion. Briefly, plasmids encoding Melan-A/MART-1 and NY-ESO-1 were respectively opened with *Xba*I and *Apa*I or *Sph*I and *Not*I before digestion with exonuclease III. This treatment was performed with the Erase-a-base System (Promega, Madison, WI).

Transfection of COS-7 cells

Transfection was performed by the DEAE-dextran-chloroquine method (14, 15). Details of the procedure have been described previously (16).

*Determination of relative affinity (RA) and stability of HLA-B*3501-identified peptides*

BM36.1 cell line, an EBV-transformed lymphoma cell line, is deficient in TAP function and expresses HLA-B*3501 mRNA. These cells (10^5/ml) were incubated overnight with peptide concentrations ranging from 10^{-16} to 10^{-4} M in serum-free RPMI 1640 medium supplemented with 100 ng/ml of β_2-microglobulin at 37°C. Subsequently, cells were stained with the B1.23.2 Ab to measure surface expression of HLA-B/C. The HLA-B*3501-binding peptide 37F (LPFDFTPGY) was used as a reference peptide (17, 18). The RA is determined as: concentration of each peptide/concentration of the 37F peptide that induces 20% of maximal HLA B/C expression (19).

For stability analysis, BM36.1 cells were incubated overnight with 10^{-4} M of each peptide, then incubated with brefeldin A (10 μg/ml) for 1 h to block cell surface expression of newly synthesized HLA-B*3501 molecules, washed, and incubated at 37°C for 0, 30 min, 1 h, 2 h, 4 h, or 6 h. Subsequently, cells were stained with the B1.23.2 Ab to evaluate the HLA-B/C molecule expression. The dissociation complex (DC50) was determined as the time required for the loss of 50% of the HLA-B*3501/peptide complexes stabilized at $t = 0$ h.

RT-PCR for tyrosinase, MAGE-A3, MAGE-A6, and NY-ESO-1 Ags

Total RNA was extracted by the guanidinium-cesium chloride procedure. Reverse transcription was performed, as previously described (11). PCR amplification was then performed on 50 ng of the cDNA with PCR buffer, 1.5 mM $MgCl_2$ (Life Technologies, Corgy Pontoise, France), 0.8 mM dNTP mix, 1 μM primers, and 0.1 U of *Taq* polymerase (Life Technologies-BRL) in a final water volume of 25 μl. Primers and PCR cycles are detailed in Table I. A total of 10 μl of PCR products was size fractionated on a 1% agarose gel. Expected lengths were 604 bp for Melan-A, 372 bp for gp100, 383 bp for tyrosinase, 615 bp for NY-ESO-1, 804 bp for MAGE-A3, and 288 bp for MAGE-A6.

Analysis of T cell responses by measurement of TNF release

A total of 10^4 TIL clones was stimulated in duplicate cultures by target cells: melanoma cells (3×10^4), transfected COS-7 cells (48 h later), or BM36.1 cells loaded with 10 μM of peptide. Culture supernatants were harvested 6 h later and tested for TNF content by a biological assay, as previously described (16). To enhance the cell surface expression of HLA molecules, melanoma cells were preincubated 48 h in medium containing 500 U/ml of IFN-γ (Tebu, Paris, France).

Cytotoxicity assay

Target cells were labeled with 100 μCi $Na^{51}CrO_4$ (Oris Industrie, Gif-sur-Yvette, France) for 1 h at 37°C before loading with synthetic peptides for 20 min. A total of 10^3 target cells was then mixed with 10^4 effector T cells (in a final volume of 100 μl), and radioactivity was measured, 4 h later, on a beta plate counter (EG&G Wallac, Evry, France).

*Generation of Melan-A-specific CTL from HLA-B*3501 PBL*

PBL were isolated from leukapheresis from healthy HLA-B*3501 volunteers. Dendritic cells (DC) were generated from adherent cells cultured for 7 days in the presence of 500 IU/ml GM-CSF and 100 IU/ml IL-4 (Abcys, Paris, France) in complete RPMI medium. On day 7, maturation agents, poly(I:C) (Sigma-Aldrich, Oakville, Canada) at 100 μg/ml and TNF-α (Abcys) at 10 ng/ml, were added in the culture for 48 h. Mature DC were loaded with peptides (5×10^{-5} M), in presence of 5 μg/ml of β_2-microglobulin, at 37°C in serum-free medium for 2 h, and then irradiated (35 Gy). A total of 3×10^6 CD4-depleted autologous PBL was then stimulated by 3×10^5 peptide-pulsed DC, in complete medium supplemented with 1000 U/ml IL-6 and 5 ng/ml IL-12 (Abcys). Irradiated DC were added again twice, at 7-day intervals in medium containing 10 U/ml IL-2 (Chiron, Amsterdam, The Netherlands) and 5 ng/ml IL-7 (Abcys). Seven days after the third stimulation, the specificity of stimulated culture was tested by intracellular IFN-γ labeling.

Analysis of T cell responses by measurement of IFN-γ production

A total of 10^5 stimulated PBL was incubated 6 h with 2×10^5 stimulator cells (peptide-pulsed BM36.1 cells or melanoma cells) in 200 μl of RPMI 1640 containing 10% FCS and 10 μg/ml brefeldin A (Sigma-Aldrich). For intracytoplasmic IFN-γ staining, cells were fixed for 10 min, washed, and stored at 4°C until staining. Negative controls were performed with unpulsed BM36.1 cells or B35-negative melanoma cell line. Lymphocytes were stained for 30 min at room temperature with the anti-human IFN-γ

Table I. *Primers and PCR cycles for Ag expression*

Ag	Sense and Antisense Primers	PCR Cycles
Melan-A	5′-CTGACCCTACAAGATGCCAAGAG-3′ 5′-ATCATGCATTGCAACATTTATTGATGGAG-3′	1 min, 94°C 1 min, 60°C 1 min, 72°C × 24
gp100	5′-AGTTCTAGGGGGCCCAGTGTCT-3′ 5′-GGGCCAGGCTCCAGGTAAGTAT-3′	2 min, 94°C 2 min, 60°C 2 min, 72°C × 30
Tyrosinase	5′-GGATAGCGGATGCCTCTCAAAG-3′ 5′-CCCAAGGAGCCATGACCAGAT-3′	1 min, 92°C 1 min, 65°C 1 min, 72°C × 25
NY-ESO-1	5′-GCCATGCAGGCCGAAGGC-3′ 5′-CTGGCCACTCGTGCTGGGA-3′	1 min, 92°C 1 min, 61°C 2 min, 72°C × 35
MAGE-A3	5′-TGGAGGACCAGAGGCCCCC-3′ 5′-GGACGATTATCAGGAGGCCTGC-3′	1 min, 94°C 2 min, 72°C × 30
MAGE-A6	5′-GATCTTCAGCAAAGCTTCCGA-3′ 5′-AGCTTCTTGGGATCCCCG-3′	1 min, 92°C 1 min, 58°C 1 min, 72°C × 30

mAb (BD PharMingen, San Diego, CA), at the concentration of 5 μg/ml in PBS containing 0.1% BSA and 0.1% saponin (Sigma-Aldrich). After two washes, cells were incubated with $F(ab')_2$ of goat anti-mouse IgG (Bio-Atlantic, Nantes, France). After staining, cells were resuspended in PBS, and 10^4 events were analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences, Grenoble, France). T cell responses were considered significant when the fraction of IFN-γ-positive cells was greater than 0.2% (after subtraction of the background obtained on control cells).

Results

MAA-derived peptides recognized by TIL clones from HLA-B35 melanoma patients are efficiently presented by HLA-B35 melanoma cell lines

Melanoma TIL, specific for Melan-A, gp100, tyrosinase, NY-ESO-1, and MAGE-A3/A6 Ags in the HLA-B35 context, had been evidenced in a previous study (11). We now used TIL-derived clones to identify the recognized epitopes. Fig. 1*A* shows the reactivity of the TIL clones to COS-7 cells cotransfected by the MAA and the restricting HLA-B35 allele.

To assess the potential interest of the epitopes recognized by these clones as targets for immunotherapy, we analyzed their presentation by a panel of melanoma cell lines. As shown in Fig. 1, *B*, *D*, and *F*, all HLA-B*3501 melanoma cell lines expressing Melan-A, tyrosinase, and MAGE-A3/A6 were recognized at a significant level by the corresponding T cell clones, and the efficiency of Ag recognition increased after IFN-γ treatment. Melanoma lines M125 and M140, lacking spontaneous expression of HLA-B*3501, were also recognized by these clones upon IFN-γ treatment.

The gp100 (Fig. 1*C*)- and NY-ESO-1 (Fig. 1*E*)-specific clones respectively recognized one of three and one of two melanoma lines (M147 and M47) that expressed the Ags and the HLA-B*35 spontaneously. IFN-γ treatment increased the efficiency of Ag recognition of these cell lines and induced that of the two cell lines lacking spontaneous expression of HLA-B*3501 (M125 and M140). As shown by RT-PCR, the two cell lines M47 and M131 that failed to be recognized by the gp100-specific clone expressed this Ag at a low level (Fig. 1*C*).

Therefore, all the epitopes recognized by HLA-B35 TIL clones were efficiently presented by most melanoma cell lines that spontaneously expressed the HLA and the MAA.

Identification of MAA-derived peptides recognized by TIL clones from HLA-B35 melanoma patients

To pinpoint the cDNA regions coding for each epitope, COS-7 cells were cotransfected by both HLA-B35 cDNA and various Ag-encoding cDNA fragments. As shown in Fig. 2, the C-terminal ends of the specific peptides were encoded by: the Melan-A cDNA

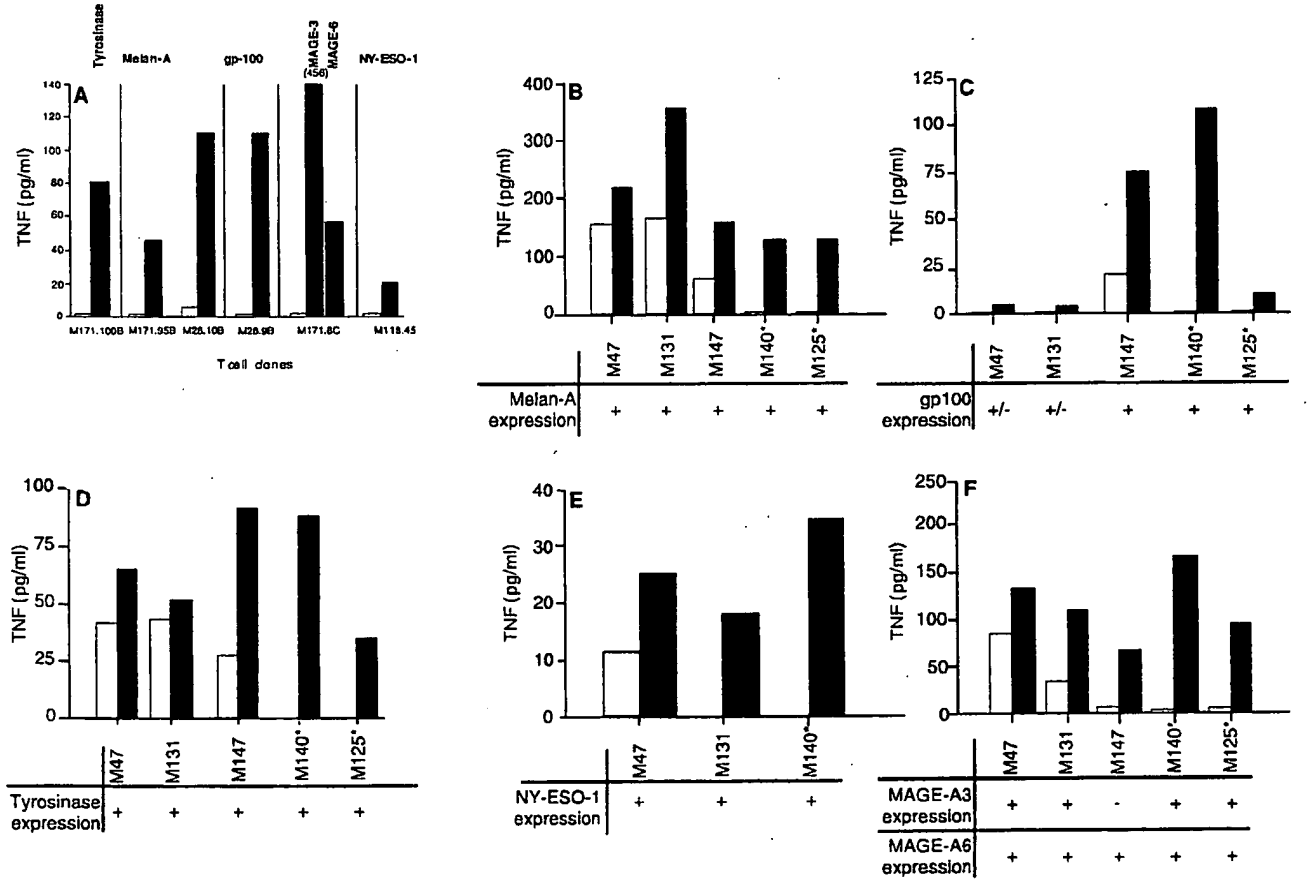

FIGURE 1. *A*, TIL clone responses to COS-7 cells cotransfected with HLA-B*3501 cDNA and a cDNA encoding the Ag. Forty-eight hours after transfection, cells were incubated 6 h at 37°C with TIL clones, and TNF secreted in the supernatant was measured by assessing its cytotoxicity for WEHI 164 clone 13. ■, TNF secretion by TIL clones in response to COS-7 cells cotransfected with cDNA encoding the HLA and the Ag. □, TNF secretion by TIL clones in response to COS-7 cells transfected with the cDNA encoding the HLA molecule alone. Recognition of B35-melanoma cells by TIL clones specific for Melan-A (*B*), gp100 (*C*), tyrosinase (*D*), NY-ESO-1 (*E*), and MAGE-A3/A6 (*F*) Ags. TIL clones were incubated for 6 h at 37°C with melanoma cell lines at an E:T ratio of 1:3. Before biological assay, melanoma cell lines were either treated (■) or not (□) with 500 U/ml of IFN-γ. TNF secreted in the supernatant was measured by assessing its cytotoxicity for WEHI 164 clone 13. In tables are indicated the level of Ag expression by melanoma cell lines as determined by RT-PCR, as described in *Materials and Methods*.

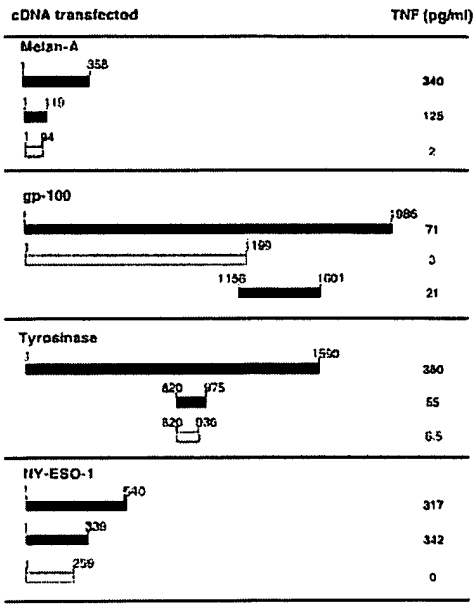

FIGURE 2. Restriction of the cDNA regions coding for the potential epitopes. Melan-A and NY-ESO-1 fragments were obtained by exonuclease digestion; the gp100 and tyrosinase fragments were obtained by PCR. Filled boxes represent fragments recognized by specific TIL clone. Those represented by open boxes are not recognized. The positions are indicated in base pairs. Fragments were cotransfected into COS-7 cells with the appropriate HLA cDNA. Forty-eight hours after transfection, cells were incubated 6 h at 37°C with TIL clones, and TNF secreted in the supernatant was measured by assessing its cytotoxicity for WEHI 164 clone 13.

Table II. *TIL clone responses to synthetic peptides*

Peptide Position	Sequence	TNF[a] (pg/ml)
Melan-A		
23–35	TAEEAAGIGILTV	107
26–37	EAAGIGILTVIL	125
26–35	EAAGIGILTV	182
27–35	AAGIGILTV	4
26–34	EAAGIGILT	13
gp100		
470–479	QVPLDCVLYR	430
471–479	VPLDCVLYR	170
471–480	VPLDCVLYRY	380
Tyrosinase		
309–322	TPRLPSSADVEFCL	308
309–321	TPRLPSSADVEFC	214
309–320	TPRLPSSADVEF	605
309–319	TPRLPSSADVE	89
309–318	TPRLPSSADV	0.2
310–322	PRLPSSADVEFCL	27
312–320	LPSSADVEF	7.4
MAGE-A3		
168–176	EVDPIGHLY	265
MAGE-A6		
168–176	EVDPIGHVY	43
NY-ESO-1		
92–104	LAMPFATPMEAEL	345
92–100	LAMPFATPM	87
94–102	MPFATPMEA	259

[a] Peptides were tested in a TNF release assay. TNF secretion of specific T cell clones was measured after 6 h of coculture with HLAB*3501 EBV cells loaded with 10 μM of peptide.

fragment 94–119 bp, the gp100 cDNA fragment 1199–1601 bp, the tyrosinase cDNA fragment 936–975 bp, and the NYESO-1 cDNA fragment 259–339 bp.

Then, using the BIMAS database for HLA class I epitope prediction, we searched the appropriate cDNA fragments for deduced peptide sequences exhibiting good HLA-B*3501-binding scores, especially those matching the known HLA-B*3501-binding motifs, i.e., P, A, or V at position 2, Y, F, M, L, or I at C-terminal position (18). Recognition of the selected peptides by the clones was then tested in chromium release assays.

The Melan-A-specific CTL clone recognized three overlapping Melan-A peptides (Table II). The peptide 26–35 (EAAGIGILTV) was the most efficiently recognized with a half-maximal lysis of 5×10^{-10} M (Fig. 3 and Table III). This peptide was also recognized by a TIL clone from another melanoma patient (data not shown).

The gp100-specific clone recognized three overlapping peptides located between aa 470 and 480 (Table II). Peptide titration analysis showed that the best fitting peptide was the decamer 471–480: VPLDCVLYRY, with a half-maximal lysis at 2×10^{-8} M (Fig. 3 and Table III). This peptide was also recognized by a TIL population derived from another melanoma patient (data not shown).

As shown in Table II, the tyrosinase-specific clone recognized three overlapping peptides: the 14-mer peptide 309–322, the 13-mer peptide 309–321, and the 12-mer peptide 309–320. Deletion of the phenylalanine at the COOH-terminal end or of the threonine at the N-terminal end of the 12-mer strongly reduced the recognition by the CTL (Table II). The 12-mer peptide was the best fitting peptide, with a half-maximal lysis at 2×10^{-10} M (Fig. 3 and Table III). Curiously, the tyrosinase nonapeptide 312–320, previously shown to be recognized by a TIL clone in the B*3501 context (20), failed to be recognized by our CTL clone (Table II and Fig. 3).

The MAGE-A3/A6-specific clone M171.8C recognized the MAGE-A3/B*3501-restricted epitope EVDPIGHLY, previously described by the group of T. Boon (21), with a half-maximal lysis at 10^{-12} M. This clone also reacted with the MAGE-A6 peptide EVDPIGHVY, albeit with a considerably lower efficacy (half-maximal lysis at 10^{-8} M) (Fig. 3 and Table III).

As shown on Fig. 4, the NY-ESO-1-specific TIL clone efficiently recognized the peptides 92–104 and 94–102 (Table II and Fig. 3). The best fitting one was clearly the nonamer 94–102 (half-maximal lysis: 10^{-12} M) (Fig. 3 and Table III).

Fine characterization of identified peptides

With the perspective to use the identified peptides for immunotherapy, their RA and stability for the HLA-B*3501 molecule were addressed, as described in *Materials and Methods*. We can distinguish two main groups: peptides having both a high affinity (RA <3) and a good stability (>6 h), and peptides having both low affinity (RA >3) and stability (<6 h). All the peptides efficiently recognized by specific TIL clones belonged to the first group, with the exception of Melan-A, which belongs to the second group (Table III).

Identification of Melan-A peptide analogues of improved affinity and stability for HLA-B35 molecule

Inefficient binding to HLA-B35 of the Melan-A peptide might be due to the lack of the main anchor residues in this peptide. Anchor residues, i.e., P at position 2 and/or Y at the N-terminal end (22), were therefore introduced to enhance the affinity of this peptide. As shown in Fig. 4, the peptides EAAGIGILTY (EAA10Y) and EPAGIGILTY (EPA10Y) exhibited both a high RA and stability for HLA-B*3501 molecule. Furthermore, these two peptides induced half-maximal lysis by the clone at concentrations respectively 50 and 10 times lower than the natural peptide. In contrast,

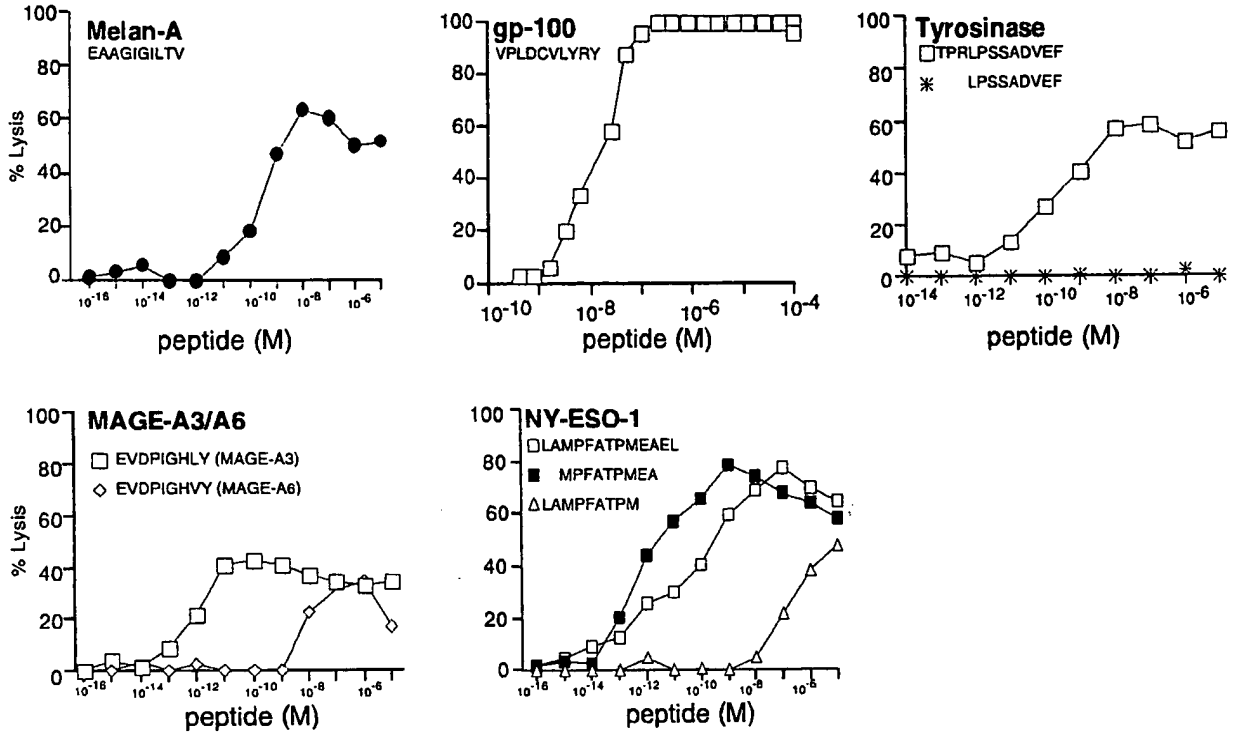

FIGURE 3. Titration analysis of peptides recognized by HLA-B35-restricted TIL clones. Target cells were a TAP-deficient cell line BM36.1 expressing the HLA-B*3501 loaded with the indicated synthetic peptides at various concentrations. Lytic activity was measured, at an E:T ratio of 10:1, by the classical 4-h ^{51}Cr release assay at 37°C.

single amino acid-substituted peptide EPAGIGILTV (EPA) did not show improved properties compared with the natural decamer.

Melan-A peptide analogues, EAA10Y and EPA10Y, are more efficient than the natural peptide in inducing the growth of Melan-A-specific CTL

CD4-depleted PBL from two HLA-B35 healthy donors were stimulated by autologous DC pulsed with the Melan-A peptide 26–35, and with the modified analogues EAA10Y and EPA10Y. Representative data obtained in one patient are shown in Fig. 5*A*. Stimulation by the natural peptide induced the growth of Melan-A-specific CTL in 13 of 48 microcultures, while modified peptides induced the growth of higher fractions of specific T cells in 36 of 48 microcultures for EAA10Y, and 42 of 48 microcultures for EPA10Y (Fig. 5*A*). Interestingly, microcultures stimulated by the Melan-A-modified peptides were also strongly reactive against the

Table III. *RA, stability of HLA/peptide complexes, and half-maximal lysis induced by each peptide*

Peptides	RA[a]	DC_{50}[b]	EC_{50}[c]
Melan-A			
EAAGIGILTV	5.9	1 h 30 min	5×10^{-10} M
gp100			
VPLDCVLYRY	2.6	>6 h	2×10^{-8} M
Tyrosinase			
TPRLPSSADVEF	1.3	>6 h	2×10^{-10} M
LPSSADVEF	1.2	6 h	
MAGE-A3			
EVDPIGHLY	2.3	>6 h	10^{-12} M
MAGE-A6			
EVDPIGHVY	1.1	6 h	10^{-8} M
NY-ESO-1			
LAMPFATPMEAEL	0.6	>6 h	10^{-10} M
MPFATPMEAEL	0.4	>6 h	10^{-12} M
LAMPFATPM	5.1	1 h 30 min	10^{-7} M

[a] The RA of each peptide is determined as the concentration of each peptide/concentration of a reference peptide that induces 20% of maximal HLA B/C expression.

[b] The dissociation complex (DC_{50}) was determined as the time required for the loss of 50% of the HLA-B*3501/peptide complexes stabilized at t = 0 h.

[c] Half-maximal lysis (EC_{50}) was the peptide concentration required to induce 50% of lysis by the specific T cell clone.

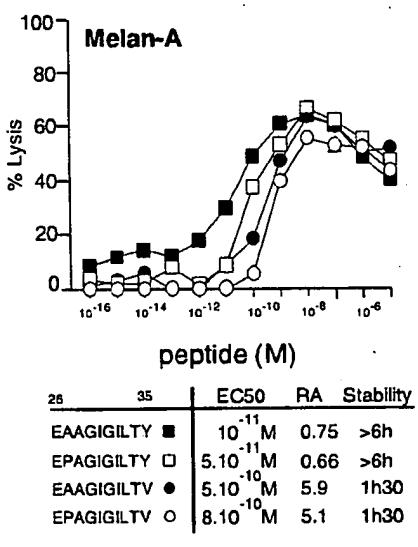

FIGURE 4. Titration analysis of natural and analog peptides recognized by the Melan-A-specific TIL clone. Target cells were a TAP-deficient cell line BM36.1 expressing the HLA-B*3501, loaded with natural and analogue Melan-A peptides at various concentrations. In table are indicated the RA, the stability of HLA/peptide complexes, measured as described in *Materials and Methods*, and the half-maximal lysis induced by each peptides.

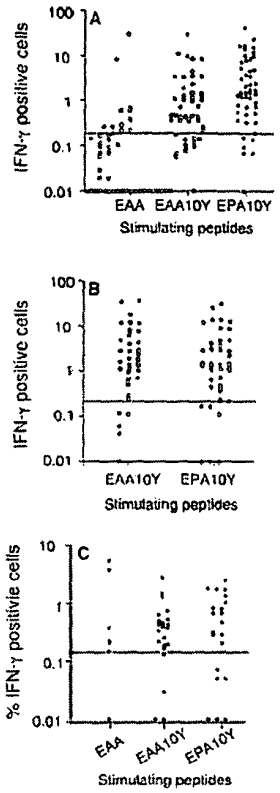

FIGURE 5. Generation of Melan-A-specific lymphocytes from PBMC. PBMC from HLA-B*3501 healthy donor were stimulated with DC pulsed either with 50 μM of natural Melan-A peptide (EAA) or with two analogue peptides: EAA10Y and EPA10Y. *A*, After three rounds of stimulation, IFN-γ secretion was measured in response to BM36.1 cells pulsed with 50 μM of the indicated peptides. *B*, IFN-γ secretion by lymphocytes stimulated by the modified peptides was measured in response to BM36.1 cells pulsed with 50 μM of the natural peptide. *C*, IFN-γ secretion of stimulated lymphocytes in response to a melanoma cell line expressing both Melan-A and the B*3501 molecule (M131). Effector and target cells were incubated at a 1:3 ratio in the presence of brefeldin A before being fixed, permeabilized with saponin-stained anti-IFN-γ Ab, and analyzed on a FACScan (10^4 cells).

natural Melan-A peptide 26–35 (Fig. 5*B*). As shown in Fig. 5*C*, most microcultures reactive against the natural Melan-A peptide also reacted against a HLA-B35 melanoma cell line expressing the Melan-A Ag.

Discussion

In this study, we described five new melanoma epitopes, recognized by CTL in association with the B*3501 molecule. To define which peptides will be the best candidates for immunotherapy in the HLA-B35 context, we analyzed the antigenicity of each identified peptide, i.e., affinity for the HLA-B35 molecule, stability of the resulting complexes, and the expression of naturally processed Ags by melanoma cells.

The B*3501-restricted Melan-A/MART-1 epitope was the peptide 26–35, also presented in the HLA-A*0201 context (Fig. 6). Because the same TIL clone also recognized this peptide in HLA-B*3503 and B*3508 contexts, this peptide could therefore be presented in at least four different HLA class I contexts. In vitro stimulations of HLA-B35 healthy donor PBL yielded Melan-A-specific T cells in a large proportion of culture wells (Fig. 5, and data not shown). This suggests that a B*3501-restricted repertoire as large as the A*0201-restricted one could exist for this peptide. Nonetheless, as shown previously for the HLA-A*0201 context (23), this peptide did not bind efficiently to the HLA-B*3501 molecule, due to a lack of appropriate anchor residues. Hence, we designed two modified peptides, EAA10Y and EPA10Y, by introducing anchor amino acids at positions 2 and/or 10. Those peptides bind more efficiently to the HLA-B*3501 and were more efficiently recognized by the specific TIL clone than the natural peptide. Moreover, in vitro generation of tumor-reactive CTL from HLA-B*3501 PBL stimulated by these peptide analogues was much more efficient than that observed with the natural peptide (Fig. 5). Because Melan-A is expressed by 90% of primary melanomas and 80% of metastatic ones, the modified Melan-A peptides are good candidates for peptide-based vaccine trials in HLA-B*35 melanoma patients.

The gp100 peptide 471–480 was the natural peptide the most efficiently recognized by the TIL clone. Although several gp100-derived epitopes presented in HLA-A or C contexts (24–26) are known, this is the first description of a HLA-B-restricted gp100 epitope. Because gp100 is a very frequently expressed MAA, this epitope also is an interesting candidate for melanoma vaccine trials. Moreover, this peptide encompasses another epitope presented in the common HLA-A*0101 context (Fig. 6, and Moreau et al., in preparation). It is therefore possible that using this peptide might stimulate T cell responses against gp100 in both HLA contexts.

The previously described B*3501-restricted tyrosinase epitope 312–320 (20) was not recognized by our tyrosinase-specific clone (Table II and Fig. 2). The longer tyrosinase peptide 309–320 was the peptide best recognized by the specific CTL clone. It is also the peptide that bound the most stably to the HLA-B35 molecule. Therefore, it is likely the natural epitope, although two longer peptides, 309–322 and 309–321, were also recognized efficiently (Table I). Other examples of 11-mer T cell epitopes have been described (27, 28). Because this peptide encompasses the previously described B*3501-restricted tyrosinase epitope 312–320 (20), it also looks an interesting candidate for peptide-based vaccination of HLA-B35 patients.

Concerning NY-ESO-1, we identified three overlapping peptides encompassed between aa 92 and 104 recognized by the TIL clone. Two of these (92–104 and 94–102) bound efficiently to the HLA-B*3501 molecule and were also recognized very efficiently by our TIL clone. Because the 94–102 peptide gives a better titration response and because it could be present as a contaminant peptide in 92–104 peptide preparation, this nonamer is most likely the natural epitope. Nonetheless, both peptides have interesting properties: the 94–102 is also presented in the HLA-B51 context (29) (Fig. 6), while the peptide 92–104 encompasses both this B51-restricted

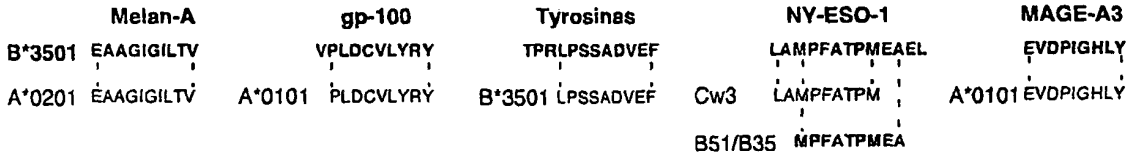

FIGURE 6. Summary of HLA-B35-restricted identified peptides. Immunization with peptides presented or encompassing shorter peptides presented in other HLA contexts could allow to vaccine simultaneously against MAA in various common HLA contexts, such as A*0201, A*0101, B51, and Cw3.

epitope and a HLA-Cw3-restricted epitope (30). In vitro PBL stimulation will be necessary to establish whether these two peptides are good candidates to stimulate melanoma-reactive CTL.

The MAGE-A3 peptide 168–176 described previously (21) was the peptide recognized by the MAGE-A3/MAGE-A6-specific TIL clone. This peptide had been identified as the target of MAGE-A3-specific PBL obtained after stimulation with DC infected with a virus coding for MAGE-A3 protein. Our results show the immunogenicity of this epitope in vivo. The same TIL clone also recognized the MAGE-A6 peptide 168–176, differing from the MAGE-A3 epitope by 1 aa in position 8, and a MAGE-A3-negative/MAGE-A6-positive HLA-B35 melanoma cell line. The MAGE-A6 peptide 168–176 is therefore a new B*3501-restricted melanoma epitope.

The present study extends to eight the number of known MAA epitopes shared by a majority of HLA-B*3501 melanoma tumors (20, 21, 31). Interestingly, several of these epitopes encompass shorter peptides that can be presented in the same or other common HLA contexts. This panel of melanoma epitopes represents an interesting pool to check the efficiency of multiepitope peptide-based vaccine protocols. Furthermore, the definition of many peptides presented by various HLA molecules allows the development of new tools for monitoring the induction of T cell responses in immunized patients. Indeed, identification of antigenic peptides is essential not only to increase the availability and efficacy of peptide-based vaccines, but also to monitor CTL responses in patients immunized with peptides or with other forms of Ags, such as full-length recombinant proteins or recombinant viruses.

Aside from these important implications for cancer immunotherapy, the observations made in this study confirm and extend previous findings concerning the ability of certain antigenic peptides to bind to more than one MHC class I gene product. It thus appears that this peptide behavior is more common than it had been anticipated previously. One may speculate that this might be the result of central tolerance that deleted T cells for high affinity MHC class I-binding peptides. Moreover, this recurrence of antigenic peptides being recognized in different MHC class I context may also reflect a bias in the Ag-processing machinery in tumor cells. Be it as it may, these findings should be taken into account when making prediction of MHC-binding peptides with currently used algorithms.

Acknowledgments

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